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**Title:** Conjugated linoleic acid isomers exert differential effects on an adipocyte model of HIV-associated lipodystrophy

**Running title:** Conjugated linoleic acid and HIV-associated lipodystrophy

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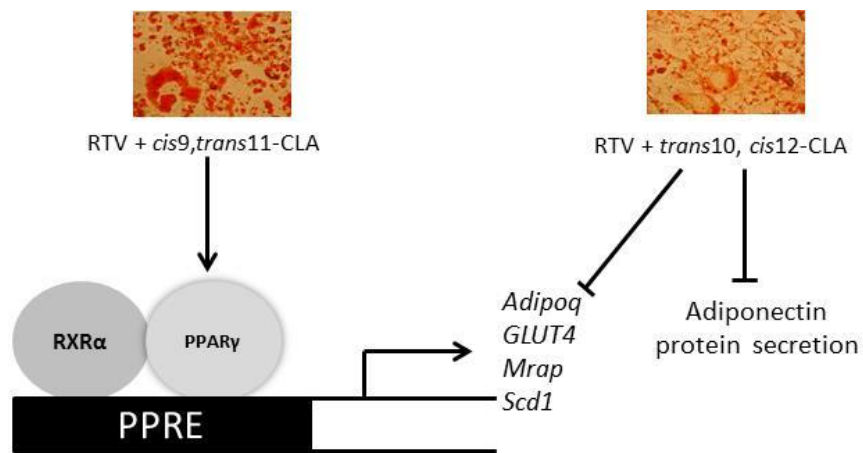
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## Graphical abstract



CLA conjugated linoleic acid; PPAR Peroxisome proliferator-activated receptor; PPRE Peroxisome proliferator-activated response element; RTV ritonavir; RXR retinoidX receptor.

→ activation

⊥ repression

## Abstract

**Background:** HIV-associated lipodystrophy is associated with decreased expression of PPAR- $\gamma$  in adipose tissue. Conjugated linoleic acid (CLA) isomers (*cis*9, *trans*11 and *trans*10, *cis*12) are putative PPAR- $\gamma$  agonists, but have not previously been investigated in the context of HIV-associated lipodystrophy.

**Methods:** 3T3-L1 pre-adipocytes were differentiated in the presence of ritonavir (20  $\mu$ M as per previous experimental models) and 100  $\mu$ M *cis*9,*trans*11, *trans*10,*cis*12 or vehicle control, DMSO. Microarray analysis, RT-PCR, DNA binding ELISA and Oil Red O staining were used to investigate adipocyte gene expression and binding, protein secretion and triglyceride storage.

**Results:** *trans*10,*cis*12 + ritonavir altered the expression of 2160 gene transcripts greater than 1.5-fold compared with control, while 257 gene transcripts were altered by *cis*9,*trans*11 + ritonavir ( $P < 0.001$ ). *trans*10,*cis*12 + ritonavir down-regulated *Pparg* (-1.55) and *Adipoq* (-2.95), as well as differentiation (*Fcor* (-4.78-fold), *Arl4a* (-4.84), *Itga6* (-2.45), *Id4* (-2.01)) and triglyceride storage genes (*Mrap* (-8.25), *Scd1* (-4.34), *Lipin1* (-2.52)). Changes in *Adipoq* were confirmed by RT-PCR ( $P = 0.038$ ) and adiponectin secretion by ELISA ( $P = 0.003$ ). *cis*9,*trans*11 + ritonavir increased PPAR- $\gamma$  nuclear binding to its gene response element ( $P = 0.038$ ). Both isomers increased triglyceride storage in the presence of ritonavir ( $P < 0.001$  and  $P = 0.001$ , respectively).

**Conclusion:** In the presence of ritonavir, *trans*10, *cis*12 appears to be detrimental, while *cis*9, *trans*11 was beneficial and may mediate its effects via PPAR- $\gamma$ . Further research is required to determine the potential role of CLA isomers as therapeutic agents in the management of HIV-associated lipodystrophy.

**Keywords:** antiretroviral therapy; conjugated linoleic acid; HIV-associated lipodystrophy; PPAR- $\gamma$ ; ritonavir

## Introduction

Conjugated linoleic acid (CLA) consists of positional and stereo-isomers of linoleic acid formed by bacterial biohydrogenation of linoleic acid in the rumen [1]. The *cis*9, *trans*11 (c9,t11) and *trans*10, *cis*12 (t10,c12) isomers are the most biologically active [1]. CLA isomers have been shown to decrease adipocyte size and lipid accumulation [2], as well as metabolic parameters including triglyceride (TG), non-esterified fatty acid, glucose and insulin concentrations [3]. The anti-obesity effect of CLA isomers has been extensively investigated, yet results are inconclusive. This may relate to the fact that c9,t11 and t10,c12 are often provided in combination, yet they exert differential effects on adipocyte gene expression [4-6]. t10,c12 appears to be the most potent isomer and decreases lipid accumulation, while c9,t11 has been shown to promote lipid accumulation [7]. The differential effects of the isomers may be mediated by PPAR- $\gamma$ , as t10,c12 has been shown to down-regulate expression of the transcription factor [4], while c9,t11 is a putative agonist [8-12].

HIV-associated lipodystrophy syndrome (HALS) is the most prevalent sub-type of acquired lipodystrophy and is associated with HIV infection and the use of highly active antiretroviral therapy (HAART) [13]. HAART consists of a combination of antiretroviral drugs (ARVs), typically chosen from one of three main drug classes: nucleoside reverse transcriptase inhibitors (NRTI), protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) [14]. Ritonavir (RTV) is a PI routinely used as a boosting agent to increase the plasma concentrations of other PI, thereby reducing their dose and frequency of use, and increasing clinical efficacy [15].

HALS manifests in different subtypes, which include lipoatrophy of the face and limbs, and lipohypertrophy of the abdomen. Additionally, morphological abnormalities also occur such as dyslipidaemia, insulin resistance and impaired glucose tolerance, hypertension, endothelial dysfunction, and altered cytokine and adipokine production [16]. The adverse effects associated with HAART affect up to 83% of treated individuals [17] and increase the risk of premature cardiovascular disease [18]. Use of RTV is associated with greater adverse effects [19]. *In-vitro* RTV decreases PPAR- $\gamma$  gene expression in subcutaneous adipocyte cultures [20] and alters the expression and secretion of adiponectin, glucose metabolism genes and pro-inflammatory cytokines [21]. We have previously investigated the effect of physiological concentrations of RTV in 3T3-L1 adipocytes and identified significant down-regulation of gene transcripts involved in adipocyte differentiation, including PPAR- $\gamma$  and adiponectin, compared with control [22].

The potential role of CLA isomers in mitigating the detrimental effects of RTV has not previously been investigated *in vitro*, nor has it been investigated *in vivo* in patients with HIV-associated lipodystrophy. This novel study aimed to determine the effect of CLA isomers on adipocyte function and gene expression in an *in-vitro* model of HIV-associated lipodystrophy with the overall goal of establishing mechanisms, which may be further investigated with a view to therapeutic implementation of CLA supplementation *in vivo*.



## 2. Materials and Methods

### 2.1. Cell culture and treatment

Murine 3T3-L1 adipocytes were chosen for the current experiments as they show homology to human adipocytes. 3T3-L1 cells exhibit properties typical of adipocytes *in vivo*, such as insulin-stimulated glucose uptake, lipogenesis, catecholamine-stimulated lipolysis and adipokine secretion. Microarray analysis has previously been carried out in 3T3-L1 cells in the context of inflammation [23] and triglyceride synthesis [24]. Additionally, our group [22] and others [21, 25, 26, 27] have previously investigated the effect of ARVs in 3T3-L1s including microarray analysis. The effect of CLA has also been investigated using this murine model [4, 28, 29]. Therefore, the 3T3-L1 cells were considered an appropriate model for investigating the effect of both ARV and CLA on adipogenic processes. 3T3-L1 preadipocytes were grown to confluence and induced to differentiate as previously described [22]. After d 2 of differentiation, the medium was removed and replaced with growth medium supplemented with insulin (1 µg/ml) and  $C_{max}$  concentrations of RTV (20 µM; VWR International, Lutterworth, UK), vehicle control (0.1% ethanol) with or without 100 µM CLA isomers (c9,t11 or t10,c12; VWR International) or fatty acid vehicle control, DMSO. Medium was changed every second day thereafter until the adipocytes were fully differentiated at d 5, as determined by light microscopy. Cells were exposed to the RTV and CLA for a total of five days. Cell viability was assessed using the MTS Cell Titer Aqueous One Solution assay on d 5 of adipocyte differentiation as previously described [22]. Cell viability was confirmed for all treatment groups prior to experimental work. Quadruplicate cell cultures were used for each experimental condition.

### 2.3 RNA extraction and preparation

Total RNA (1 µg) was extracted from adipocytes after 5 days of exposure to antiretroviral drug, CLA isomer or control using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Manchester, UK). Briefly, cells were lysed and homogenised using a highly denaturing guanidine-thiocyanate-containing buffer. Ethanol was added to provide appropriate binding conditions and the sample was then applied to an RNeasy Mini spin column. Total RNA binds to the membrane and contaminants were efficiently washed away. RNA was eluted in 50 µl nuclease-free water. RNA was quantified using the NanoDrop® 1000 3.7.1 (Thermo Scientific, Wilmington, USA). RNA integrity numbers can be found in Supplementary Table 1.

### 2.4 Microarray hybridisation and data analysis

Microarray hybridisation was carried out as previously described using Affymetrix GeneChip® Mouse Genome 430 2.0 Array [22]. The array contained 45,102 probe sets representing 41,975 genes, of which 34,000 are well-substantiated mouse genes.

RNA samples were pooled to minimise individual variation as a source of gene-expression variance (one pool representing four control-treated cells, one representing the four c9,t11+RTV-treated cells and one representing the four t10,c12+RTV-treated cells). 500 ng of each of the three pooled samples in 5 µl nuclease-free water was used in the reactions.

Data were analysed using the Robust Multi-array Average approach using quantile normalisation and the mean change in expression was determined for each gene using the formula  $\text{=IF}(\text{'ratio value'} < 1, -1/\text{'ratio value'}, \text{'ratio value'})$  where the ratio value is ratio of treatment/control. Differentially expressed genes were defined using a threshold of 1.5-fold relative to control (signal log ratio  $\geq 1.0$  or  $\leq -1.0$ ). MetaCore™ pathway analysis software

(GeneGo, Thomson Reuters, New York) was used to identify differentially expressed genes and to classify these genes into clusters based on functionality.

A list of relevant gene ontology (GO) terms describing genes affected by RTV in terms of their biological function was generated using MetaCore™ software. Affymetrix probe identifications showing a difference of greater than 1.5-fold between RTV+c9,t11- or RTV+t10,c12-treated cells and controls were added and a P value was generated based on the number of interactions each gene had within the pathway. A false discovery rate (FDR) of 1 was selected to identify all objects with at least one connection with the dataset. Microarray data are archived with the NCBI Gene Expression Omnibus repository (series record GSE70854).

## *2.5 Real-time RT-PCR*

cDNA was generated by reverse transcription using the High Capacity RNA-to-cDNA kit (Invitrogen Life Technologies) as per manufacturer's instructions. Real-time RT-PCR was used to measure PPAR- $\gamma$  (Mm01184322\_m1), adiponectin (Mm00456425\_m1) and endogenous housekeeping control hypoxanthine-guanine phosphoribosyltransferase (Hprt; Mm 1545399\_m1) gene expression using TaqMan® Gene Expression Assays (Invitrogen Life Technologies). Data were analysed using the  $2^{-\Delta\Delta CT}$  method for target gene expression normalised to control [30].

## *2.6 ELISA*

Adiponectin concentration in cell culture media was determined on d 5 of differentiation. Cell supernatants were extracted, centrifuged at 12,000 rpm for 10 min, aliquoted and stored at -20°C. Adiponectin concentration in supernatants was determined using the DuoSet®

ELISA Development kit as per manufacturer's protocol (R&D Systems Inc., Abingdon, UK).

All samples were analysed in duplicate.

## *2.7 Nuclear PPAR- $\gamma$ activation*

Nuclear proteins were extracted from adipocytes on d 5 of differentiation using a nuclear extract kit as per manufacturer's protocol (Active Motif, Rixensart, Belgium). The Bradford assay was used to determine protein concentration as per manufacturer's instructions (Sigma Aldrich). 10  $\mu$ g of nuclear extract from differentiated adipocytes on d 5 of differentiation was used in a TransAM™ PPAR- $\gamma$  transcription factor assay (Active Motif, Rixensart, Belgium) to investigate PPAR- $\gamma$  nuclear binding to the peroxisome proliferator response element (PPRE) as per manufacturer's instructions. Absorbance was measured on the Synergy HT-1 spectrophotometer (Biotek, UK) at 450 nm with a reference wavelength of 655 nm.

## *2.8 Oil Red O staining*

TG accumulation at d 5 of differentiation was determined using ORO staining (Sigma Aldrich). Briefly, cells were fixed with 10% formalin for 1 h, rinsed with 60% isopropanol and stained with 60% ORO working solution prepared in isopropanol for 10 minutes. After washing with distilled water, the ORO was eluted using 100% isopropanol and the optical density of the solution measured at 540 nm using the Synergy HT-1 spectrophotometer (Biotek). Adipocyte images were captured by using a DXM 1200 Digital Camera (Nikon, Kingston Upon Thames, UK) and a Lucia G Image-Processing System (version 4.61; Nikon).

## *2.9 Statistical analysis*

Statistical analyses were performed using SPSS software (version 20; IBM, Hampshire, UK).

Data for RT-PCR, ELISA and DNA binding ELISA were analysed using independent

samples t-tests to compare treatment (RTV + t10,c12 or RTV + c9,t11) with control (RTV + DMSO). Differences were considered significant if  $P < 0.05$ . Data are expressed as the mean  $\pm$  standard error of the mean (SEM).

### 3. Results

#### 3.1. Microarray analysis: c9,t11 and t10,c12

All probe sets which produced an “absent” or “no change” signal compared with control were removed. Of the remaining probes, those with greater than a 1.5-fold change compared with control were selected in order to minimise background noise and isolate only significantly altered gene transcripts.

For t10,c12-treated cells, these probes represented 2160 gene transcripts with altered expression, of which 1209 (56 %) were down-regulated and 951 (44 %) were up-regulated compared to control. A full list of probe IDs for the 2160 gene transcripts altered by RTV+t10,c12 is available online (Supplementary Table 2). The results, showing the unique gene transcripts and fold changes in expression in t10,c12+ritonavir -treated compared with control cells, are also available online (Supplementary Table 3).

The addition of c9,t11 to ritonavir-treated cells resulted in 257 gene transcripts with altered expression compared with control, of which 146 (57 %) were down-regulated and 111 (43 %) were up-regulated compared to control. A full list of probe IDs for the 257 genes altered by RTV+c9,t11 is available online (Supplementary Table 4). The results, showing the unique genes and fold changes in expression in c9,t11-treated compared with control cells, are also available online (Supplementary Table 5).

##### 3.1.1. Gene ontology

The main GO processes affected by RTV+t10,c12 or RTV+c9,t11 are listed in **Figure 1** and **Figure 2** (all  $P < 0.001$  compared to control). Fat cell differentiation was the top GO process affected by RTV + t10,c12, while homophilic cell adhesion via plasma membrane adhesion molecules was the top GO process affected by RTV + c9,t11. Network objects (genes) within these two main GO processes are listed in **Table 1** with their corresponding fold changes.

### 3.1.2. Effect of CLA isomers on *Pparg* and *Adipoq* expression

The addition of t10,c12 to RTV-treated cells down-regulated *Pparg* mRNA (-1.55-fold) and *Adipoq* (-2.95-fold) mRNA transcripts in microarray analysis compared with control. In contrast, RTV + c9,t11 up-regulated *Pparg*, but only by 1.15-fold, which was not detected using the assigned 1.5-fold change cut-off. No significant effect was observed for c9,t11 + RTV on *Adipoq* mRNA transcripts.

RTV + t10,c12 down-regulated a number of gene transcripts involved in adipocyte differentiation, the main GO process affected. These include melanocortin 2 receptor accessory protein (*Mrap*; -8.25-fold), free fatty acid receptor 2 (*Ffar2*; -4.58-fold), ADP-ribosylation factor-like 4A (*Arl4a* -4.84-fold), stearoyl-Coenzyme A desaturase 1 (*Scd1*; -4.34), integrin alpha 6 (*Itga6*; -2.45-fold) and inhibitor of DNA binding 4 (*Id4*; -2.01). *Pparg* regulatory genes beta-3 adrenergic receptor (*Adrb3*; -6.36-fold) and lipin 1 (*Lpin1*; -2.52) were also down-regulated by RTV + t10,c12. Transcripts for genes involved in insulin sensitivity (foxo1 corepressor (*FCoR*; -4.78-fold; *Steap4*; -4.08-fold) and glucose metabolism (*Glut4*; -3.67-fold) were down-regulated by RTV + t10,c12. PPAR- $\gamma$  target gene and inducer of adiponectin secretion endoplasmic reticulum oxidoreductase 1-L alpha (*Ero1l*) was down-regulated by RTV + t10,c12 (-2.49-fold). Lipolysis regulatory genes inhibin beta b (*Inhbb*) and Bernardinelli-Seip congenital lipodystrophy 2 homolog were also down-regulated by

RTV + t10,c12 (-2.89-fold and -2.29-fold, respectively), as well as the energy metabolism gene SH2B adaptor protein 2 (*Sh2b2* -2.56-fold).

A number of gene transcripts were up-regulated by RTV + t10,c12 including high mobility group AT-hook 2 (*Hmga2*; + 6.96-fold), cyclin D1 (*Ccnd1*; +3.04-fold), inhibin beta-A (*Inhba*; +2.09-fold) and guanine nucleotide binding protein, alpha stimulating) complex locus (*Gnas*; +1.62-fold).

Within the main GO process affected by c9,t11 + RTV, gene transcripts from the protocadherin gamma subfamily (*Pcdhga*) were up-regulated (+1.5-fold). No gene transcripts were down-regulated greater than 1.5-fold.

### 3.2 Validation of microarray by RT-PCR

RT-PCR was used to confirm microarray findings for two genes of interest PPAR- $\gamma$  and adiponectin. In the presence of RTV, neither t10,c12 nor c9,t11 exerted a significant effect on PPAR- $\gamma$  gene expression in RTV-treated cells (**Figure 3**). Adiponectin gene expression was significantly decreased by RTV + t10,c12 compared with control ( $P = 0.038$ ; **Figure 4**). c9,t11 had no significant effect on adiponectin gene expression in the presence of RTV.

### 3.3 PPAR- nuclear binding

The effect of CLA isomers on nuclear PPAR- $\gamma$  binding to the immobilised consensus site (PPRE) was investigated in adipocytes treated with RTV. c9,t11 significantly increased nuclear PPAR- $\gamma$  binding to the immobilised consensus site in both RTV- treated cells ( $P = 0.038$ ; **Figure 5**). No significant effect was observed for t10,c12.

#### 3.4 Adiponectin secretion

The effect of the CLA isomers on adiponectin secretion was investigated in adipocytes treated with RTV. t10,c12 significantly decreased adiponectin secretion in RTV-treated cells ( $P = 0.003$ ; **Figure 6**). c9,t11 increased adiponectin by 40%, but this did not reach significance.

#### 3.5 Triglyceride accumulation

TG accumulation was investigated in 3T3-L1 adipocytes treated with RTV and CLA isomers using Oil Red O staining. Under RTV exposure, both c9,t11 and t10,c12 significantly increased in TG accumulation compared to control cells ( $P < 0.001$ ; **Figure 7A and 7B**).



#### 4. Discussion

We have previously shown that RTV alters adipocyte gene expression with detrimental consequences for the adipocyte [22]. It was hypothesized that the addition of CLA isomers to RTV-treated adipocytes would modulate some of these detrimental effects. For the first time in an *in-vitro* model of HIV-associated lipodystrophy, c9,t11 was found to have a neutral or beneficial effect, while t10,c12 exerted a detrimental effect on adipocyte differentiation and gene expression.

Binding of lipophilic ligands to PPAR- $\gamma$  leads to the transcription of adipogenic genes [31], which regulates adipogenesis and lipid metabolism [32]. Caron *et al* [33] highlight a central role for PPAR- $\gamma$  down-regulation in the etiology of HALS. In the current study, RTV + t10,c12 down-regulated PPAR- $\gamma$  mRNA transcripts, as revealed by microarray analysis. In contrast, RTV + c9,t11 increased PPAR- $\gamma$  mRNA expression, although this did not reach significance. A DNA binding assay was used to determine the level of binding of PPAR- $\gamma$  mRNA to the PPRE. Interestingly, the addition of c9,t11 to RTV-treated cells significantly up-regulated PPAR- $\gamma$  binding to PPRE, despite showing a non-significant effect at the mRNA level. Previous studies have shown increased PPAR- $\gamma$  gene expression in c9,t11-treated cells, although in the absence of RTV [9, 10]. Despite an effect at the mRNA level, t10,c12 did not significantly alter PPAR- $\gamma$  binding. Previous studies in the literature have shown decreased PPAR- $\gamma$  expression [4], mass [34], activation and protein levels [35] in response to t10,c12, but again in non-RTV-treated cells.

PPAR- $\gamma$  target gene adiponectin plays a role in insulin sensitization and its down-regulation may contribute to insulin resistance in HALS [36]. In the current study, adiponectin gene transcripts were down-regulated in adipocytes treated with RTV + t10,c12 compared with control in microarray analysis, which may be a consequence of down-regulation of PPAR- $\gamma$ . Adiponectin protein secretion was also significantly down-regulated by RTV+t10,c12

compared with control. Additionally, down-regulation of *Ero1l* may have contributed to the reduction in adiponectin mRNA by RTV + t10,c12, as *Ero1L* has been shown to increase adiponectin mRNA expression following activation by PPAR- $\gamma$  in mature adipocytes [37]. Both antiretroviral drugs [22, 38] and t10,c12 [34] alone have been shown to significantly decrease adiponectin mRNA expression, therefore, the combination of RTV + t10,c12 would be expected to result in down-regulation. In the case of RTV+c9,t11, non-significant increases in adiponectin mRNA expression (29%) and protein secretion (40%) were observed. These findings correspond with previous research using c9,t11 only in 3T3-L1 adipocytes [5] and in mice [39], and indicates the potential for c9,t11 to overcome the detrimental effects of RTV on adiponectin.

In the current study, leptin expression was not altered by RTV + CLA. Leptin is another important adipokine, typically decreased in HALS and leptin administration in HALS was found to be beneficial in improving lipoatrophy and dyslipidaemia [40, 41]. Previous studies in 3T3-L1 cells show no effect of ARVs only [22, 42] or c9,t11 only [43] on leptin expression, but t10,c12 decreased leptin expression in rats [44]. Although c9,t11 may not increase leptin expression, it appears to be involved in the other mechanisms underlying adipocyte dysfunction in HALS, including down-regulation of PPAR- $\gamma$ .

Similar to adiponectin, melanocortin 2 receptor accessory protein (*Mrap*) is transcriptionally activated by PPAR- $\gamma$  via a PPRE in the *Mrap* promoter region [45]. *Mrap* was the top gene within the main GO process (fat cell differentiation) down-regulated by RTV + t10,c12 (-8.25-fold) in the current study. *Mrap* is a G-protein coupled receptor for adrenocorticotrophic hormone [45] and *Mrap* knockdown in adipocytes is associated with increased lipid droplet formation, but no change in PPAR- $\gamma$  expression [46]. In the current study, we observed a decrease in *Mrap* mRNA transcripts and PPAR- $\gamma$  mRNA transcripts, and an increase in TG accumulation, which corresponds with findings from the study of Betz *et al* and provide a

possible explanation for the increase in TG accumulation observed in RTV+t10,c12-treated cells. Neither t10,c12 nor RTV have previously been investigated in terms of their effect on Mrap expression.

Following differentiation, lipid metabolism genes orchestrate changes in the adipocyte, which result in the accumulation of triglyceride within lipid droplets. Lipin-1 (*Lpin1*) is a transcriptional coactivator of such lipid metabolism genes and was down-regulated by RTV+t10,c12 in the current study. In our previous study, RTV down-regulated *Lpin1* expression [22] and in the current study t10,c12 was unable to counteract this effect. Down-regulation of *Lpin1* may play an important role in the development of adipose tissue abnormalities in HIV as highlighted by data showing significantly lower *Lpin1* mRNA expression in subcutaneous adipose tissue from patients with HALS compared with those without HALS [47, 48]. The lipotrophic effect of RTV+t10,c12 is further demonstrated by its inhibitory effect on stearoyl-Coenzyme A desaturase 1 (*Scd1*), as well as anti-lipolytic genes inhibin beta b (*Ihbb*) and Bernardinelli-Seip congenital lipodystrophy 2 homolog (*Bscl2*).

In the present study, RTV+c9,t11 did not exert a detrimental effect on adipocytes when compared with RTV+t10,c12. The main GO process altered by c9,t11 was homophilic cell adhesion by plasma membrane adhesion molecules and genes from the protocadherin gamma subfamily were the only genes altered and were up-regulated within this GO process. Protocadherin gamma is one of a family of cell adhesion proteins and forms part of a homophilic subset of Pcdhg isoforms with specific roles in intercellular interactions [49]. To the best of the authors' knowledge, Pcdhg has not previously been shown to be altered by either RTV or CLA isomers.

Triglyceride accumulation was investigated to determine whether changes in mRNA expression of lipid metabolism genes translated to changes in the adipocyte phenotype. RTV

has been shown to decrease adipocyte TG accumulation [22, 50], and in the current study the addition of c9,t11 and t10,c12 to RTV-treated cells significantly increased TG accumulation compared with control. c9,t11 has not previously been shown to affect TG accumulation, while evidence from the literature strongly supports the anti-lipogenic effect of t10,c12 [4, 7, 26, 51-53]. However, it is important to bear in mind that these studies investigated CLA isomers alone, without antiretroviral drugs. Down-regulation of Mrap gene transcripts as discussed above, may have contributed to the increase in TG accumulation. Additionally, it could be suggested that the effects of t10,c12 may differ depending on prevalent conditions such as the presence of RTV. This has been observed in a study of porcine peripheral blood mononuclear cells where the addition of an endotoxin to cells treated with t10,c12 reversed the effects of the isomer on NF- $\kappa$ B activity and TNF- $\alpha$  expression [54]. Therefore, it is possible that the presence of RTV in the current study reversed the normal adipocyte response to t10,c12.

At present, the majority of CLA supplements commercially available consist of either a mixture of CLA isomers or of the t10,c12 isomer alone, while c9,t11 is the main isomer found naturally in meat and dairy products [55]. The feasibility of CLA supplementation in HIV-infected individuals has previously been investigated in a study carried out by our group. In this study, a mixed c9,t11 and t10,c12 supplement was used (Clarinol A80, Stepan Lipid Nutrition Ltd., Koog aan de Zaan, The Netherlands) at doses of 3 g per day for 12 weeks. Due to the small sample size, this study has not been published, but details of the trial can be found on the ISRCTN clinical trial registry (DOI 10.1186/ISRCTN31131566).

Limitations of the current study include the fact that RTV was investigated at 20  $\mu$ M only; however, *in vivo*, circulating RTV levels are not sustained i.e. levels peak and trough. Future studies using different drug concentrations and over different time points would help to determine the potential effects of RTV at a range of therapeutic levels. Secondly, the use of

murine adipocytes limits the generalizability of the study and future studies with primary human adipocytes would enhance the clinical relevance of these findings. Additionally, no combinations of c9,t11 and t10,c12 were tried in the current study, as the focus of this initial work was to elucidate the effects of each isomer individually. Future studies should investigate the effect of the isomers in combination, given that both isomers increased TG accumulation, but only the t10,c12 isomer caused detrimental effects. The c9,t11 isomer is the most abundant dietary form, while commercial supplements typically consist of significant amounts of t10,c12 [56]. As each isomer was investigated separately, our experimental set-up used a common *in vitro* concentration. Although the dose used does not reflect plasma concentrations following dietary or supplementary CLA, it is within the range expected within adipocytes following dietary supplementation [2]. Future studies should also investigate RTV+c9,t11 in the presence of a PPAR- $\gamma$  inhibitor such as GW9662, to determine whether the effects on TG accumulation occur via a PPAR- $\gamma$ -dependent mechanism.

## Conclusion

For the first time this study demonstrates an effect of CLA isomers in a model of HIV-associated lipodystrophy. CLA isomers exerted differential effects on mRNA expression of a number of genes including PPAR- $\gamma$  and adiponectin, but both isomers increased TG accumulation. Although, the effect of t10,c12 on TG accumulation may be viewed as potentially beneficial in the context of HIV lipoatrophy, the inhibitory effect of t10,c12 in the presence of RTV on key adipogenic genes argues against the use of t10,c12 in combination with antiretroviral drugs. The findings indicate the potential of the c9,t11 isomer to modulate PPAR- $\gamma$  and potentially alleviate ARV-induced down-regulation of PPAR- $\gamma$ , an important mechanism underlying HALS. Further research is required to explore this and to determine

369 whether c9,t11 should be pursued as a potential therapeutic agent in HIV-associated  
370 lipodystrophy.

371

#### 372 **Conflict of interest**

373 The authors declare no conflict of interest at any stage in the preparation or publication of  
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376

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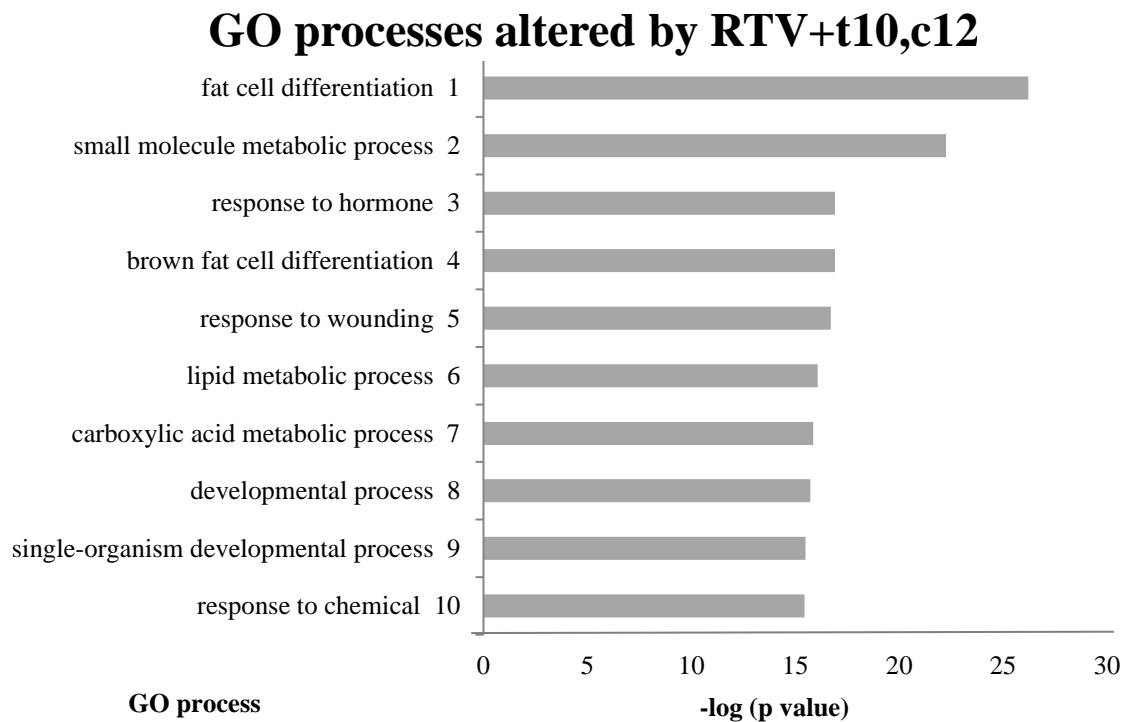
378 The authors are grateful to Dr Emilie Stolarczyk for providing the 3T3-L1 adipocytes used in  
379 this study.

380

381

## Tables and figures

**FIGURE 1**

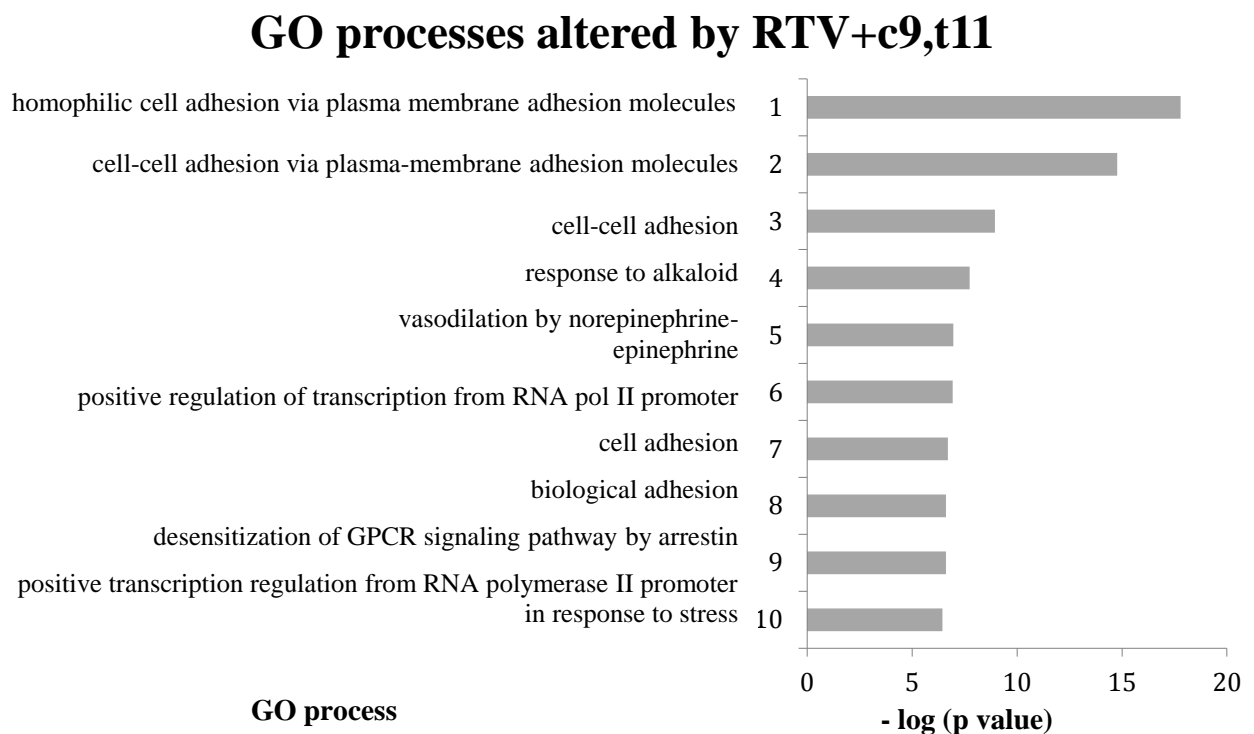


**Figure 1** Gene Ontology processes affected by RTV + t10,c12-CLA. Significant representation in Gene Ontology probes indicated a change in gene expression between RTV+t10,c12 and control cells. These genes were categorised according to the gene ontology processes they formed part of. The Gene Ontology Terms significantly represented are shown. The length of each bar is proportional to the ratio of the number of genes altered compared with the total number of genes in the GO network.  $P < 0.001$  for all Gene Ontologies shown.





**FIGURE 2**



391 **Figure 2** Gene Ontology processes affected by RTV + c9,t11-CLA. Significant  
 392 representation in Gene Ontology probes indicated a change in gene expression between  
 393 RTV+c9,t11 and control cells. These genes were categorised according to the gene ontology  
 394 processes they formed part of. The Gene Ontology Terms significantly represented are  
 395 shown. The length of each bar is proportional to the ratio of the number of genes altered  
 396 compared with the total number of genes in the GO network.  $P < 0.001$  for all Gene  
 397 Ontologies shown.

**Table 1** Gene probes altered within the main GO processes

<b>t10,c12 + RTV</b>			
<b>Fat cell differentiation: down-regulated genes</b>			
<b>Probe identification</b>	<b>Gene name</b>	<b>Gene symbol</b>	<b>Fold change</b>
1451371_at	melanocortin 2 receptor accessory protein	<i>Mrap</i>	-8.25
1449182_at	resistin	<i>Retn</i>	-8.10
1455918_at	adrenergic receptor, beta 3	<i>Adrb3</i>	-6.36
1435092_at	ADP-ribosylation factor-like 4A	<i>Arl4a</i>	-4.84
1439834_at	Foxo1 corepressor	<i>FCoR</i>	-4.78
1425215_at	free fatty acid receptor 2	<i>Ffar2</i>	-4.58
1415965_at	stearoyl-Coenzyme A desaturase 1	<i>Scd1</i>	-4.34
1460197_a_at	STEAP family member 4	<i>Steap4</i>	-4.08
1450699_at	selenium binding protein 1	<i>Selenbp1</i>	-3.81
1415958_at	Glucose transporter type 4	<i>Glut4</i>	-3.67
1434484_at	WAP four-disulfide core domain 21	<i>Wfdc21</i>	-3.43
1422651_at	adiponectin, C1Q and collagen domain containing	<i>Adipoq</i>	-2.95
1426858_at	inhibin beta-B /// similar to Inhbb protein	<i>Inhbb</i> /// <i>LOC100046802</i>	-2.89

1428333_at	Signal peptidase complex subunit 3	<i>SPC22</i>	-2.70
1450718_at	SH2B adaptor protein 2	<i>Sh2b2</i>	-2.56
1418288_at	lipin 1	<i>Lpin1</i>	-2.52
1419030_at	Endoplasmic Oxidoreductin-1-Like Protein	<i>Ero1l</i>	-2.49
1422444_at	integrin alpha 6	<i>Itga6</i>	-2.45
1437345_a_at	Bernardinelli-Seip congenital lipodystrophy 2 homolog (human)	<i>Bscl2</i>	-2.29
1423259_at	inhibitor of DNA binding 4	<i>Id4</i>	-2.01

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#### **Fat cell differentiation: up-regulated genes**

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<b>Probe identification</b>	<b>Gene name</b>	<b>Gene symbol</b>	<b>Fold change</b>
1450780_s_at	high mobility group AT-hook 2	<i>Hmga2</i>	6.96
1448698_at	cyclin D1	<i>Ccnd1</i>	3.04
1422053_at	inhibin beta-A	<i>Inhba</i>	2.09
1421740_at	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	<i>Gnas</i>	1.62

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#### **c9,t11 + RTV**

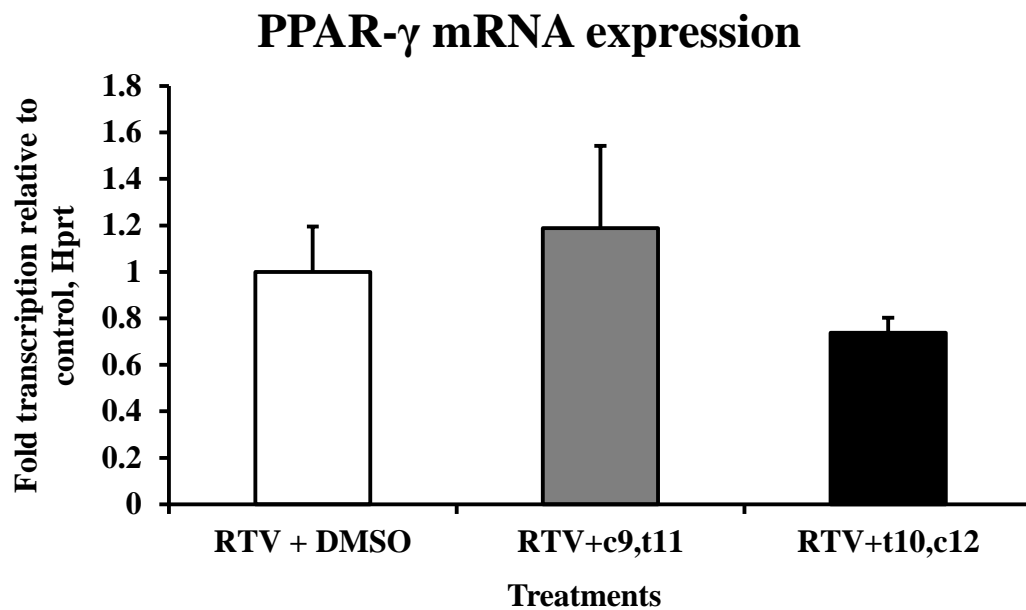
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#### **Homophilic cell adhesion via plasma membrane adhesion molecules: up-regulated genes**

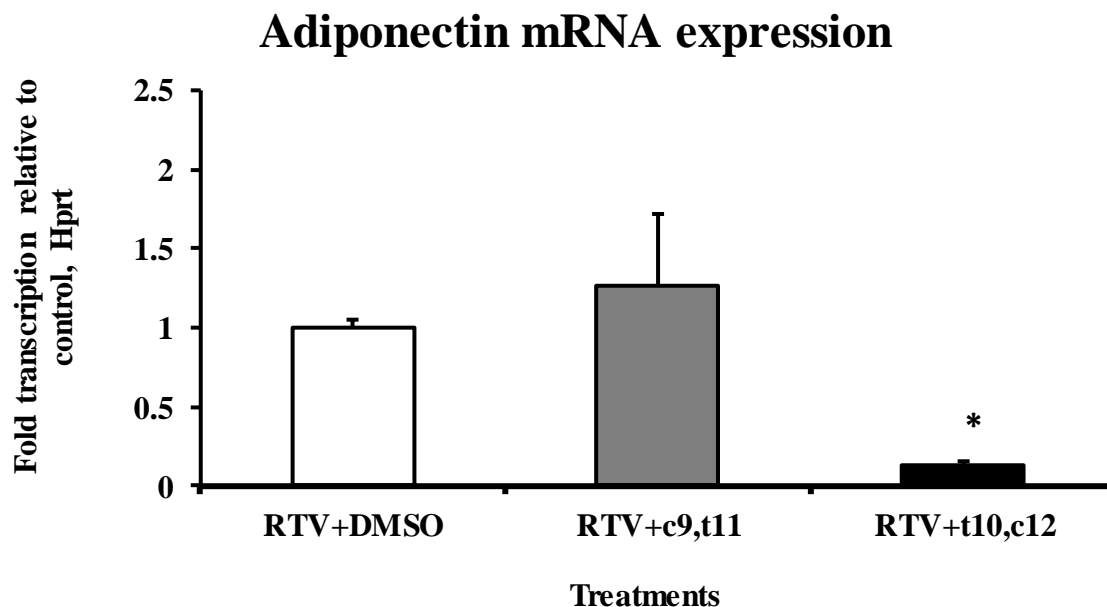
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Probe identification	Gene name	Gene symbol	Fold change
1423628_s_at	Protocadherin gamma subfamily	<i>Pcdhga</i> 1-12,	
	A1-12, B1-7, C3-5	<i>Pcdhgb</i> 1-7,	1.50
		<i>Pcdhgc</i> 3-5	

**FIGURE 3**

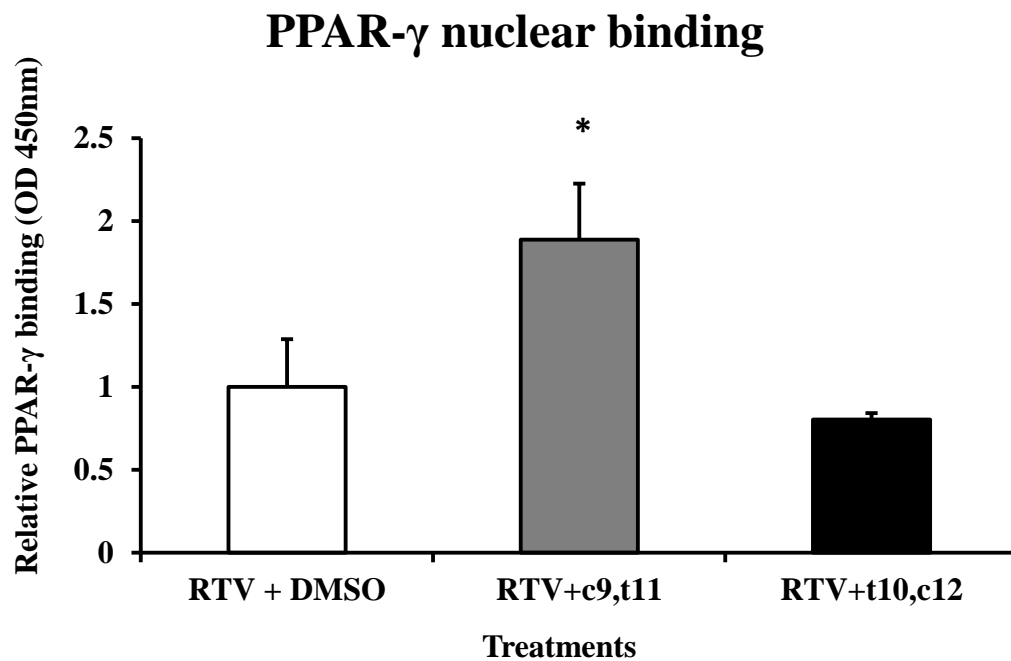


**Figure 3** Effect of RTV and CLA isomers on PPAR- $\gamma$  transcription. Adipocytes were treated with  $C_{\max}$  concentrations of RTV and DMSO or RTV and 100  $\mu$ M of c9,t11 or t10,c12. Expression of PPAR- $\gamma$  mRNA was measured on day 5 of differentiation relative to *Hprt* housekeeping gene by RT-PCR. Results represent mean  $\pm$  SEM of quadruplicate cell culture experiments. Not significantly different from control cells.



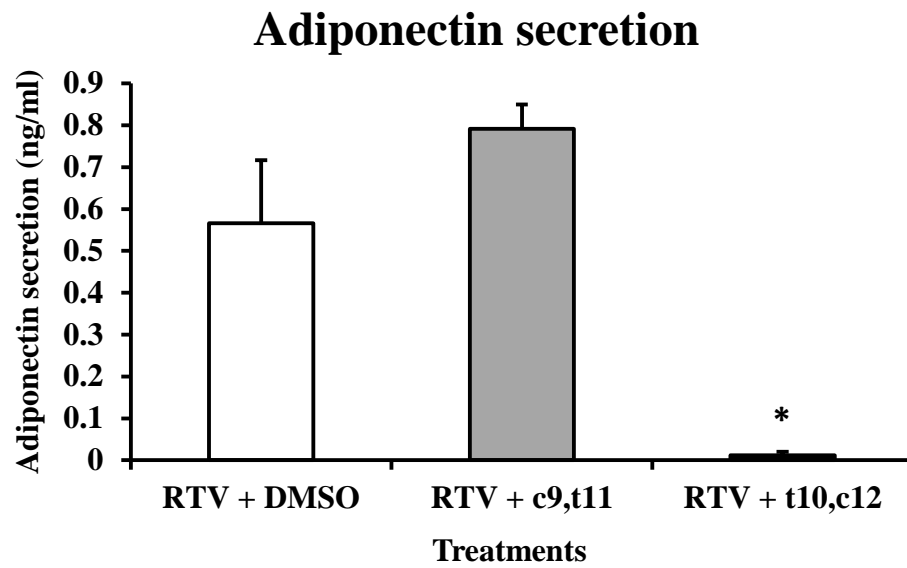
**Figure 4** Effect of RTV and CLA isomers on adiponectin transcription. Adipocytes were treated with control or  $C_{max}$  concentrations of RTV and DMSO or RTV and 100  $\mu$ M CLA isomers, c9,t11 or t10,c12. Expression of adiponectin mRNA was measured on day 5 of differentiation relative to *Hprt* housekeeping gene by RT-PCR. Results represent mean  $\pm$  SEM of quadruplicate cell culture experiments. \*Significantly different ( $P = 0.038$ ) from control cells.

**FIGURE 5**



411 **Figure 5** Effect of RTV and CLA isomers on nuclear PPAR- $\gamma$  binding to PPRE. Nuclear  
412 proteins were extracted on day 5 of differentiation from adipocytes treated with control or  
413  $C_{max}$  concentrations of RTV and DMSO or RTV and 100  $\mu$ M of c9,t11 or t10,c12 and nuclear  
414 PPAR- $\gamma$  consensus site binding was measured. Results represent mean  $\pm$  SEM of  
415 quadruplicate cell culture experiments. \*Significantly different ( $P = 0.038$ ) from control  
416 cells.

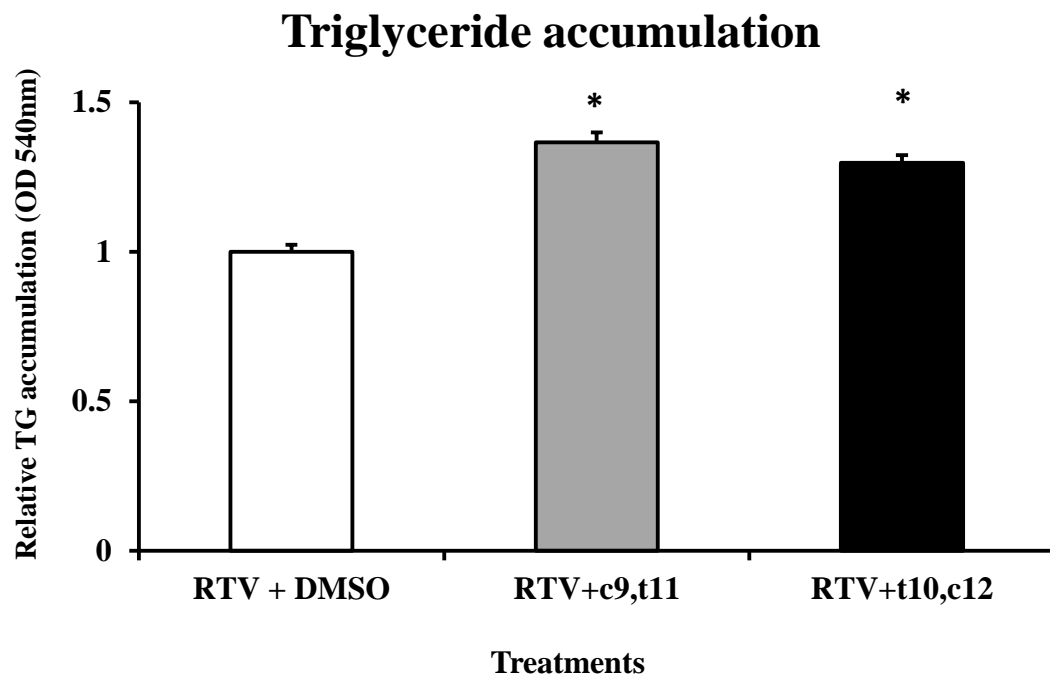
**FIGURE 6**



417 **Figure 6** Effect of RTV and CLA isomers on adiponectin protein secretion. Adipocytes were  
418 treated with control or  $C_{\max}$  concentrations of RTV and DMSO or RTV and 100  $\mu$ M CLA  
419 isomers, c9,t11 or t10,c12. Supernatants were collected from adipocytes on day 5 of  
420 differentiation and used to quantify adiponectin secretion. Results represent mean  $\pm$  SEM of  
421 quadruplicate cell culture experiments. \*Significantly different ( $P = 0.003$ ) from control  
422 cells.

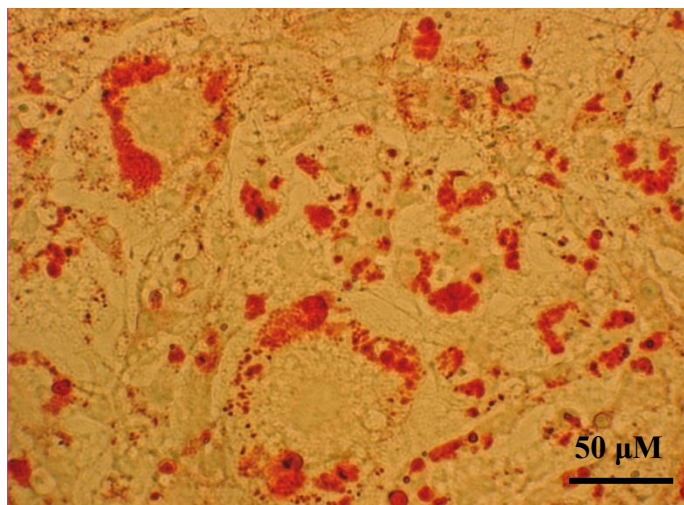


**FIGURE 7A**

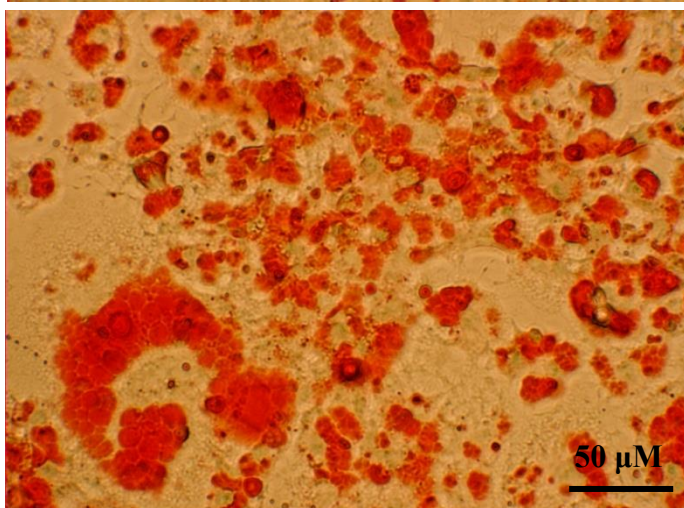


423 **Figure 7** The effects of RTV and CLA isomers on TG accumulation in 3T3-L1 adipocytes.  
424 (A) Lipid content was measured spectrophotometrically at A 540 nm and is represented as  
425 mean  $\pm$  S.E.M. of quadruplicate cell culture experiments. \* Significantly different ( $P < 0.001$ )  
426 from control cells. (B) Adipocytes treated with vehicle control (RTV+DMSO) or RTV with  
427 100  $\mu$ M c9,t11 or t10,c12 were stained at day 5 with ORO to visualize lipid content  
428 [magnification is at 40x].

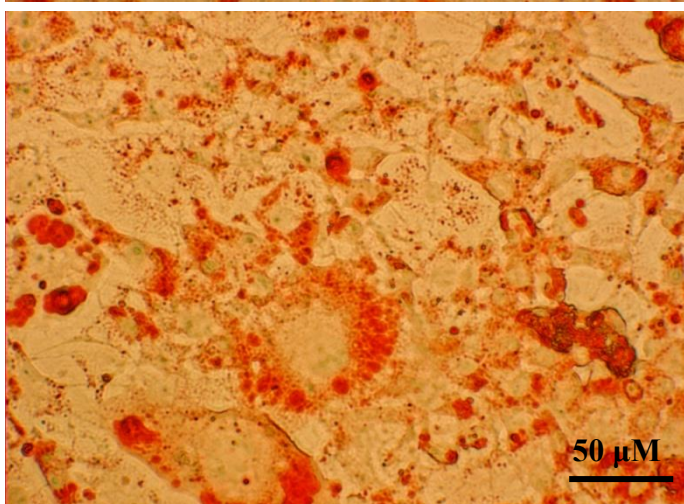
**FIGURE 7B**



**RTV + DMSO**



**RTV + c9,t11**



**RTV + t10,c12**

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